Virology **254,** 37–49 (1999)
Article ID viro.1998.9550, available online at http://www.idealibrary.com on IDEAL®

A Particulate Viral Protein Vaccine Reduces Viral Load and Delays Progression to Disease in Immunized Ponies Challenged with Equine Infectious Anemia Virus

Scott A. Hammond,* Sheila J. Cook,† Louis D. Falo, Jr.,‡ Charles J. Issel,† and Ronald C. Montelaro*,[1]

*Departments of Molecular Genetics and Biochemistry and ‡Dermatology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15261; and †Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, Kentucky 40546

Received July 8, 1998; returned to author for revision September 28, 1998; accepted November 30, 1998

Immunization regimens that induce a broadly reactive cytolytic T lymphocyte (CTL) response specific for lentiviral antigens have emerged as the leading candidates in efficacy trials conducted in both animal models and humans. To date, lentivirus vaccination strategies have overlooked one such immunization strategy, namely the use of particulate antigens. To evaluate the efficacy of targeting antigen into the phagocytic pathway to elicit a cell-mediated immune response to lentiviral antigens, we initiated the first study of a particulate-based vaccination protocol using a large animal model system. Gradient-purified equine infectious anemia virus (EIAV) was covalently coupled to glutaraldehyde-activated iron oxide beads. *In vitro* studies demonstrated the effectiveness of the inactivated whole virus particulate to prime antigen presenting cells for the activation and expansion of virus-specific $CD8^+$ CTL. The *in vivo* effectiveness of the particulate antigen was evaluated by experimental immunization of ponies. Ponies receiving the viral particulate vaccine and challenged with infectious EIAV had a delayed progression to disease and a reduced viral load compared with infected ponies that had not been vaccinated. Interestingly, *in vitro* virus-specific CTL activity was detected in only one of four immunized animals at the day of challenge. The beneficial effects of the particulate vaccine regimen were not clearly associated with any *in vitro* measurable parameters of the virus-specific cellular or humoral immune responses elicited by the vaccine at the day of challenge. However, within 3 weeks after virus challenge, anamnestic humoral responses characterized by a rapid emergence of neutralizing activity in the serum and a predominance of conformationally dependent epitopes recognized by virus-specific antibodies were observed in the vaccinates. Taken together, further studies are clearly warranted in large animal model systems using a particulate-based vaccine regimen considering the beneficial effects of this regimen in our study and the protective effects of particulate antigen delivery in the murine model.

INTRODUCTION

Equine infectious anemia virus (EIAV), a macrophage-tropic lentivirus, causes in horses a persistent infection and a chronic disseminated disease of worldwide importance (Montelaro *et al.,* 1993). The disease that develops in the host after infection by EIAV via blood-feeding insects or blood from contaminated syringe needles (Issel and Foil, 1984; Stein *et al.,* 1942) is characterized by recurring cycles of viremia and of clinical episodes characterized by fever, anemia, edema, thrombocytopenia, and various wasting symptoms (Sellon *et al.,* 1994). Among lentiviruses, EIAV is unique in that despite aggressive virus replication and rapid antigenic variation, a large majority of the infected animals progress from a chronic disease state to an inapparent carrier stage that can be immunologically maintained for life. It is interesting for the development of candidate vaccine strategies that the animals survive the infection not by completely clearing the viral infection but rather by maintaining a strict immunological control over virus replication. Thus the lentivirus model system of EIAV offers a unique advantage over other members of the lentiviral family in that immunological control of the viral infection by the host does occur and that the ability to induce a similar response by novel immunization strategies therefore should be possible. Indeed, encouraging reports of various levels of vaccine protection in the EIAV model systems have been documented (Issel *et al.,* 1992).

The development of an efficacious vaccine to control and/or prevent lentiviral infections remains a high priority in human and veterinary medicine but also remains an elusive goal. Many different antigenic preparations (purified viral protein, recombinant protein, peptides, immune stimulating complexes (ISCOMs), liposomes, and so on) and recombinant vector strategies (vaccinia virus, canary pox, adenovirus, retrovirus, and so on) have been developed and implemented using multiple animal model systems to elicit protective immunity and to identify immune correlates of protection to lentiviral infections. The current wisdom in vaccine development is that one of the key, if not the key, parameter or parameters in the host's immunological arsenal necessary for the prevention or control of lentiviral infection is a vigorous, virus-specific cytolytic T lymphocyte (CTL) response. To

[1] To whom reprint requests should be addressed at W1144 Biomedical Science Tower. E-mail: rmont@pop.pitt.edu.

0042-6822/99 $30.00

prime the host with such virus-specific CTL to prevent or control an EIAV infection, we investigated the use of a recently described novel vaccination strategy, namely targeting of antigen coupled to particulate beads into the phagocytic pathway (Rock, 1996).

Antigens covalently associated to particulate beads have been successfully used to introduce various antigens into the MHC class I restricted and, to a lesser degree, the MHC class II restricted processing and presentation pathways for the priming of a vigorous antigen-specific CTL response (Kovacsovics-Bankowski and Rock, 1994; Rock, 1996). Particulate antigen immunization has been shown to prime CTLs that can protect animals from challenge with tumors (Falo *et al.,* 1995). Exogenous antigens presented in particulate form are predominantly taken up from the environment by phagocytes (Rock, 1996), which include macrophages, the primary target cell for EIAV replication *in vivo* (Montelaro *et al.,* 1993). Direction of antigen to the predominant target of EIAV replication would seem most appropriate for the induction of a similar CTL response to that of an actively infected cell.

In this study, we covalently coupled gradient-purified EIAV virions to iron oxide beads. We evaluated the capacity of this multicomponent particulate antigen preparation to be processed and presented by phagocytic cells for recognition by virus-specific T cells *in vitro* and to elicit immunity to EIAV *in vivo*. Immunized animals were experimentally infected with EIAV to determine the *in vivo* effectiveness of the vaccine regimen. Immunological responses induced by the vaccine regimen in the animals were measured using a panel of *in vitro* humoral and cellular analyses.

RESULTS

Characterization of the infectivity of EIAV covalently coupled to particulates

We generated a preparation of gradient-purified $EIAV_{PV}$ covalently conjugated to glutaraldehyde-activated 0.5- to 1.5-μm iron oxide beads (EIAV-beads) and evaluated its potential use as a candidate vaccine to induce vigorous cell-mediated immune responses. Immunization with this particulate-based strategy required the delivery of noninfectious virus to the animals. Therefore we assessed the infectivity of EIAV covalently attached to iron oxide beads by pulsing permissive cells, macrophages, and equine dermal cells with various preparations of gradient-purified EIAV and EIAV-beads. Levels of RT activity in the culture supernatants were measured. Cultures were considered productively infected if the average measured RT activity in the supernatant of experimental cultures were >2 S.D above the RT activity measured in supernatants from uninfected cell cultures. In equine dermal cell cultures (Fig. 1A) and equine macrophage cultures (Fig. 1B), only the addition of gradient-purified EIAV initiated a productive infection. Supernatants of both types of cell cultures on the addition of EIAV-beads treated with or without formaldehyde, a known inactivating agent of EIAV (Issel *et al.,* 1992), or gradient-purified EIAV inactivated with formaldehyde were negative for RT activity (Figs. 1A and 1B).

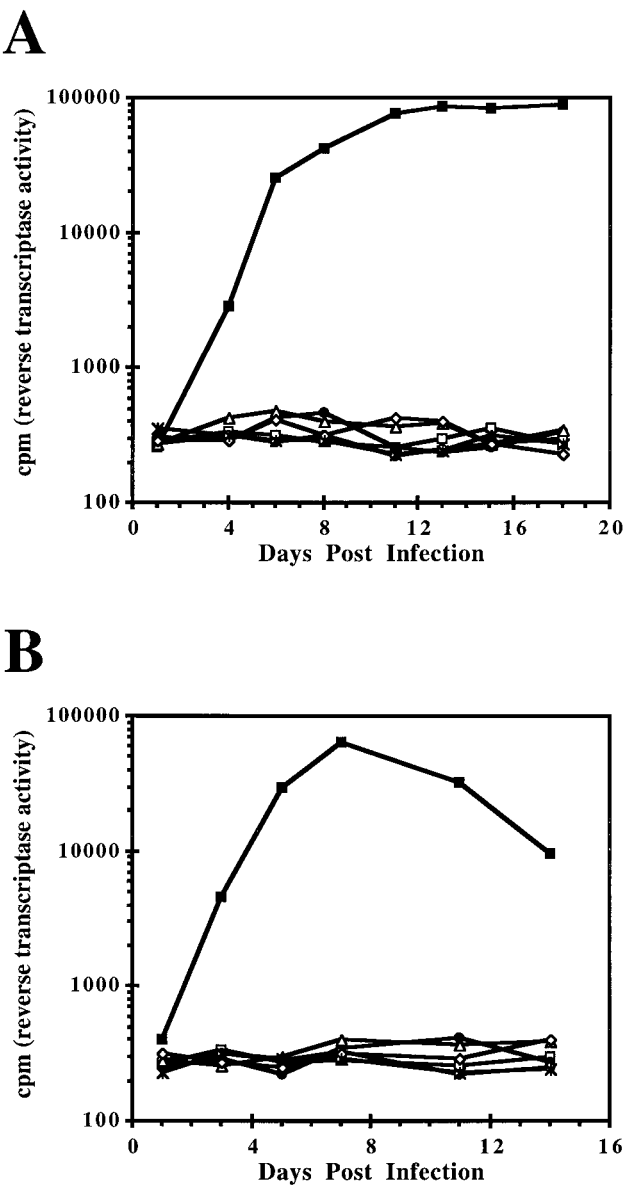

FIG. 1. Reverse transcriptase activity levels in the supernatants of equine dermal cells (A) or equine macrophages (B) exposed to infectious EIAV and EIAV covalently coupled to iron oxide beads. Cells were incubated with 3 μg/ml of gradient-purified EIAV (■), 0.8% formaldehyde treated gradient-purified EIAV (□), EIAV attached to iron oxide beads (●), EIAV attached to iron oxide beads and treated with 0.8% formaldehyde (○), EIAV attached to iron oxide beads sonicated (△), the supernatant of EIAV attached to iron oxide beads sonicated (◇), or BSA attached to iron oxide beads (*). Virus production at the various times postaddition in the supernatant of the incubated cells was measured in a micro-reverse transcriptase assay as described under Materials and Methods. Data points were conducted in triplicate. S.D.s for all data were always <15%. Data presented were representative of two separate experiments.

We next tested the possibility that infectious virions might freely dissociate from the particulate antigen preparations. EIAV-beads in PBS were briefly sonicated in an ultrasound waterbath to dissociate any virions that were not covalently associated with the iron oxide particles. The supernatant of the sonicated particulates was transferred to equine macrophages and equine dermal cells and measured for RT activity in the cell culture medium. All sonicated particulate preparations added to both cell types were negative for RT activity (Figs. 1A and 1B). Sonicated EIAV-beads supernatants were negative for protein content as measured in a Lowry-based microtiter assay and by Western blot analysis of pelleted supernatants (data not shown). Therefore EIAV-beads were determined to contain covalently bound virions that were not infectious.

In vitro cell-mediated immune responses elicited by immunization with EIAV-beads

The virus–particulate complex was compared with soluble, infectious, gradient-purified EIAV for its ability on addition to equine macrophages to elicit an *in vitro* secondary immune response by primed T cells isolated from EIAV-infected ponies. The antigen presenting cell used in this assay was the macrophage, the predominant *in vivo* target for replication of EIAV (Montelaro *et al.,* 1993). Macrophages have the capacity to process and present exogenous particulate antigens with MHC class I molecules (Rock, 1996). Autologous macrophages that had processed and presented EIAV antigens after the initiation of a productive infection by infectious gradient-purified EIAV or after pulsing with EIAV-beads effectively stimulated EIAV-specific oligoclonal T cells to incorporate [^{3}H]thymidine in a dose-dependent manner (Fig. 2A). The responding T cells in the cultures induced by the addition of gradient-purified EIAV were ~70% CD4$^+$, with some cells CD8$^+$ (10–15%) as determined by indirect immune fluorescence (data not shown). In contrast, the responding T cell cultures on addition of the particulate preparation were >65% CD8$^+$, with few (<15%) CD4$^+$ cells observed (data not shown). Maximum levels of stimulation occurred with the addition of 3–10 μg/ml of gradient-purified EIAV and 3 μg/ml of gradient-purified EIAV coupled to beads. The addition of particulate antigens at >10 μg of antigen/ml elicited toxic effects on the macrophages due to an overabundance of particles phagocytosed by the cells, which in turn resulted in a reduction in the level of the observed lymphoproliferation (Fig. 2A). Autologous macrophages pulsed with BSA conjugated to iron oxide particles did not induce any antigen-specific response by the T cells (Fig. 2A). Thus EIAV presented to macrophages in the form of a noninfectious particulate was processed and presented for recognition by predominantly virus-specific CD8$^+$ T cells.

We compared and contrasted the antigen-specific cytolytic effector functions of the responding T cell populations isolated from long-term EIAV-infected ponies to macrophages that had been pulsed with infectious gradient-purified EIAV or EIAV-beads. EIAV infected macrophages activated and expanded both CD4$^+$ *env*-specific and CD8$^+$ *gag*-, *pol*-, and *env*-specific CTL (Fig. 2B). In contrast, the macrophages pulsed with EIAV-beads activated and expanded only CD8$^+$ *gag*- and *env*-specific CTL (Fig. 2C). The lower level of *env*-specific CTL compared with the *gag*-specific CTL and the lack of *pol*-specific CTL in cultures activated and expanded by macrophages pulsed with particulate antigen was consistently observed in all replicate experiments. Thus EIAV-infected macrophages and macrophage-pulsed with EIAV-beads activated and expanded responding T cell populations that differed in antigen specificity and phenotype.

Immunization with an inactivated whole virus particulate vaccine

To evaluate the *in vivo* effectiveness of the EIAV-beads preparation to elicit a cell-mediated immune response, we initiated a vaccination protocol for which four naive outbred ponies were inoculated with the EIAV-beads preparation. Clinical evaluations were performed daily on all animals. Immunological responses were monitored at predetermined intervals after initiation of the vaccine. Both cell-mediated (EIAV-specific lymphoproliferative and CTL responses) and humoral (*env*-specific antibody quantity and quality as measured in neutralization, avidity, and conformation analyses) responses were followed.

All animals received 200 μg of EIAV-beads divided evenly on either side of the neck and administered a total of three times with 3 weeks between each inoculation. Clinical observations and temperature profiles were monitored daily, and platelet counts were monitored weekly after initiation of the particulate regimen (Fig. 3). No evidence of a pathogenic EIAV infection in the form of a temperature above 39°C, platelet count below 105,000/μl, or any clinical signs was observed before infectious virus challenge.

To further detect any productive replication of the EIAV-beads after the administration of the immunogen, we conducted two *in vitro* measurements of viremia: levels of infectious virus and copies of viral RNA present in plasma. On the day of virus challenge, no infectious virus as measured by culturing undiluted plasma onto permissive fetal equine kidney cells or viral RNA as measured by semiquantitative RT-PCR were detectable in the plasma of the vaccinated animals (Table 1). These results, together with clinical observations, confirm the lack of a productive infection in the animals receiving the EIAV-beads vaccine regimen.

Ponies were inoculated with EIAV-beads to elicit an

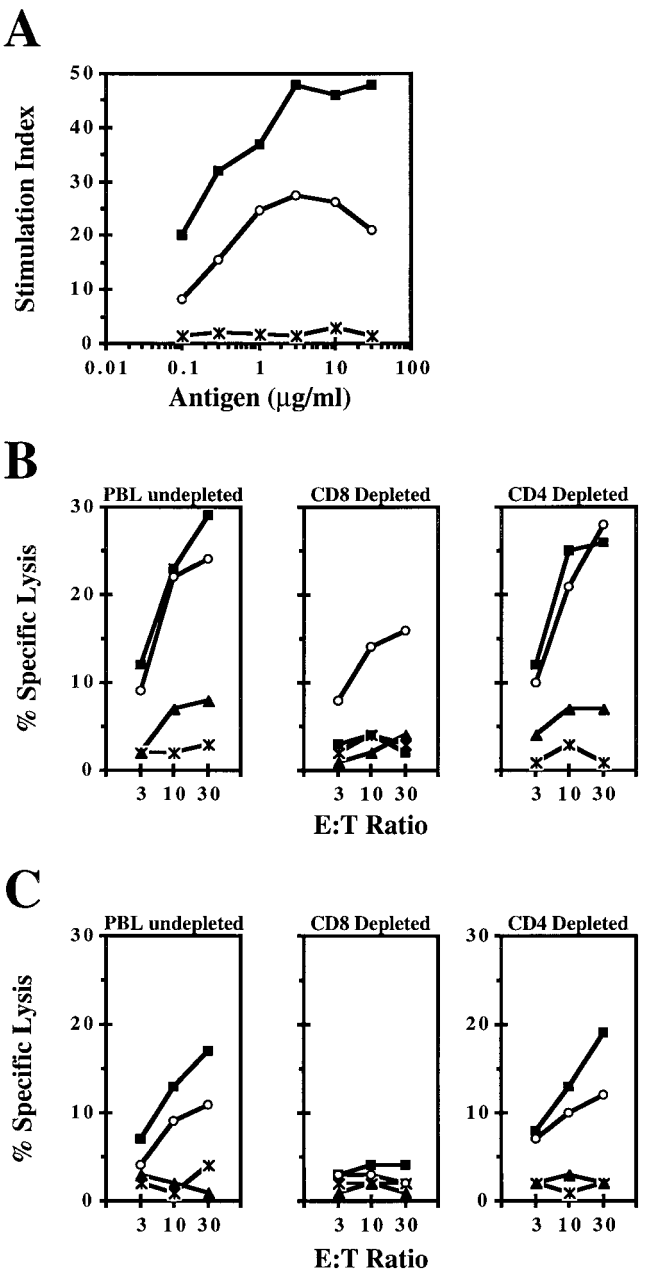

FIG. 2. *In vitro* secondary cell-mediated immune responses mediated by EIAV covalently coupled to iron oxide beads. (A) Nonadherent PBLs isolated from long-term EIAV infected ponies were mixed with autologous macrophages infected with varying amounts of gradient-purified EIAV (■), pulsed with varying amounts of EIAV attached to iron oxide beads (○), or pulsed with varying amounts of BSA attached to iron oxide beads (starbursts). Proliferation was measured by [^{3}H]thymidine incorporation 5 days postaddition and was presented in the form of an SI that represented [^{3}H]thymidine incorporation of antigen-stimulated cultures divided by [^{3}H]thymidine incorporation of unstimulated cultures. Mean incorporation of [^{3}H]thymidine of replicate unstimulated lymphocyte cultures were always <400 cpm. All experiments were conducted in quadruplicate. The graph shown was a representative of three experiments. The SI S.E.M. for each data point was always <10%, as calculated under Materials and Methods. (B and C) PBLs isolated from long-term infected ponies were activated and expanded *in vitro* with autologous macrophages infected with 10 μg/ml gradient-purified EIAV (B) or pulsed with 3 μg/ml gradient-purified EIAV attached to iron oxide particulates (C). The responding cultures were used at various effector-to-target ratios in a standard chromium release assay after no treatment (undepleted), after depletion of CD8$^+$ cells, or after depletion of CD4$^+$ cells. After depleting the specific subpopulations, effector cell addition at the various effector-to-target ratios were normalized appropriately according to the original CD4/CD8 ratios measured by flow cytometry in the undepleted effector population. Autologous target cells were generated by infection with recombinant vaccinia virus vectors carrying sequences encoding EIAV *gag* (■), *pol* (▲), *env* (○), or β-galactasidase (starbursts). Data presented is representative of 2 separate experiments. SEM for all data points was always <3%.

EIAV-specific cell-mediated immune response to evaluate the role of T cells in the control or prevention of an EIAV infection. Strong lymphoproliferative responses by both CD4$^+$ and CD8$^+$ T cells to autologous macrophages infected with EIAV were observed (stimulation indices of 15–35) at the day of virus challenge (Fig. 4). These results clearly established that the particulate vaccine generated a readily detectable cell-mediated response. Qualitative analyses of the cell-mediated response by measuring EIAV-specific CTL activity from the vaccinated subjects at day of challenge, however, detected statistically significant activity in standard chromium release assays for only one pony in the study, pony 621 (Fig. 5B). The lack of readily detectable CTL activity observed in the peripheral blood leukocytes (PBLs) collected from the vaccinates was confirmed by conducting experiments in which PBLs were activated and expanded *in vitro* with one of two methods in parallel: autologous macrophages infected with EIAV or autologous pokeweed mitogen (PWM)-stimulated lymphoblasts infected with recombinant vaccinia virus vectors carrying sequences encoding EIAV *gag*, *pol*, and *env*. To increase the potential for lytic activity to be observed, all responding effector cell populations were enriched for CD8$^+$ T cells by indirect panning or positive selection for CD8$^+$ T cells. Nonetheless, only pony 621 had any detectable cytolytic activity on target cells expressing EIAV antigens (Fig. 5B).

Quantitative and qualitative humoral immune analyses of the envelope glycoprotein-specific antibody elicited by the particulate vaccine regimen were analyzed at the day of challenge (DC) using measurements of serum antibody titer, avidity, conformation, and neutralizing capabilities (Fig. 6). Quantitative analysis of *env*-specific antibody demonstrated the presence of moderate levels (reciprocal end point titers $\sim 10^3$) of the antibody populations in all animals tested (Fig. 6A). Although *env*-specific antibody was present in all animals, we were unable to detect any neutralizing activity to autologous virus in our standard neutralization assay (Fig. 6B). Avidity index measurements of the envelope-specific antibody were extremely low (<5%) for all animals in this trial (Fig. 6C). Conformational ratio analyses of *env* antibody reactivities to native or denatured viral envelope glycoproteins demonstrated a predominance (ratios 1.7–3.3) of

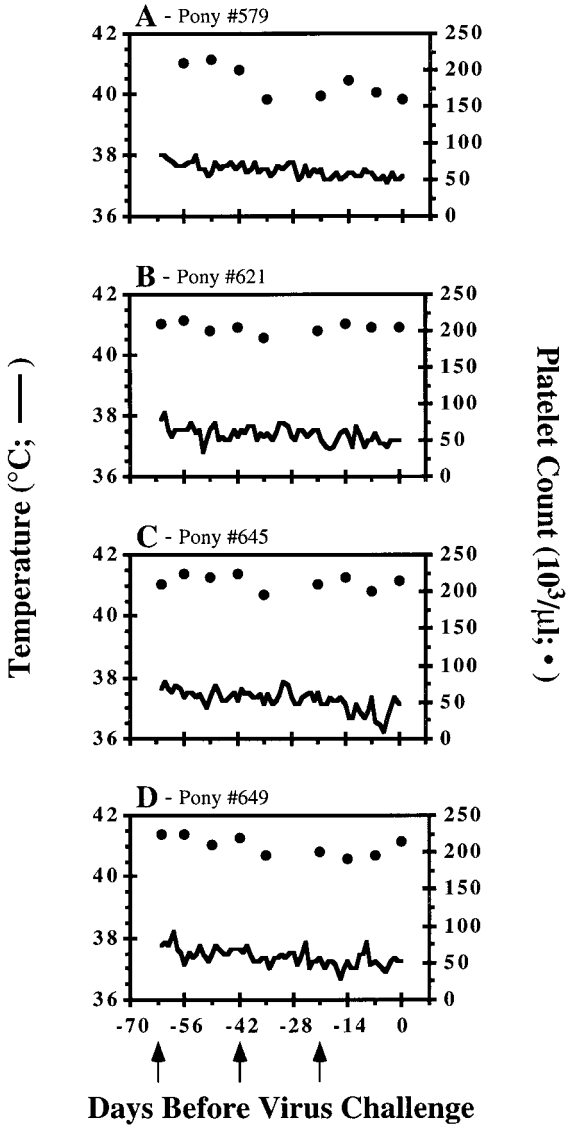

FIG. 3. Clinical profiles of ponies inoculated with EIAV-beads before challenge with infectious EIAV. Four outbred ponies (579, 621, 645, and 649) were inoculated intradermally with 200 μg of EIAV attached to iron oxide particulates at the indicated time points (arrows) before challenge with infectious EIAV. Rectal temperatures in degree centigrade were plotted using the primary *y*-axis (solid line). Platelet counts per microliter of whole blood were represented on the graphs using the secondary *y*-axis (●). Arrows indicate the days on which the ponies were inoculated with immunogen.

antibody specificity for conformation-dependent epitopes over linear epitopes (Fig. 6D). Taken together, the quantitative and qualitative measurements of the humoral immune response induced by the EIAV-beads suggested a relatively immature antibody response as described for the lentivirus model systems of EIAV (Hammond *et al.,* 1997)and SIV (Cole *et al.,* 1997).

Virus challenge of vaccinated ponies

Nonvaccinated ponies experimentally infected with $EIAV_{PV}$ progressed to clinical EIA in ~16–19 days (Figs. 7E and 7F; Table 1). Concurrent with the initial EIA-related fever was a rapid decline in the quantity of platelets circulating in the blood. In some cases of EIAV infection, ponies recrudesce with more severe clinical manifestations of high fever (>39°C) and extremely low blood platelet counts of <105,000/μl of blood (Fig. 7F). Quantities of viral RNA in excess of 10^6 copies/ml of plasma were measured in as little as 14 days postinfection from most nonvaccinated ponies experimentally infected with EIAV (Table 1). Thus in animals with a naive immune system to EIAV, this strain of EIAV induces an acute syndrome characterized by a disseminate disease that rapidly overcomes the host's immune defenses during the initial stage of infection.

In contrast to naive animals, ponies vaccinated with the EIAV-beads preparation demonstrated, in general, a delay in disease progression and a lower virus burden (Figs. 7A–7D, Table 1). All vaccinated animals were challenged with 300 equid infectious doses of $EIAV_{PV}$ 3 weeks after the final immunization. Interestingly, one animal, pony 579, did not show any clinical signs of disease during the 65 days of observations in the form of a febrile episode or a drop in platelet counts below 105,000/μl after challenge with infectious EIAV (Fig. 7A). Of the three remaining animals, two ponies, 621 and 649, had a delay in the progression to disease (44 and 58 days after virus challenge) compared with nonvaccinated and experimentally infected ponies (16–21 days after virus challenge). The single remaining pony, 645, progressed to disease at the same rate as naive animals experimentally challenged with infectious virus.

The initial day postchallenge at which infectious virus could be detected *in vitro* was delayed in the vaccinates (Table 1), from 8–13 days after virus challenge in nonvaccinates and 14 to >22 days after virus challenge in vaccinates. Comparing and contrasting the viral RNA levels using semiquantitative RT-PCR of the three immunized ponies that had a delay in disease (579, 621, and 649) to the nonimmunized ponies at 21 days after virus challenge resulted in, on average, 1000-fold less viral RNA in the vaccinates than the control animals (Table 1). Statistical analysis comparing the virus RNA levels at day 21 postchallenge in animals receiving the particulate vaccine with a panel of nonvaccinated animals (Table 1) using a nonparametric, two-tailed Mann-Whitney test resulted in a value of $P < 0.01$. This result emphasized the positive effects of the particulate vaccine by providing a statistically significant correlation between the reduction in virus burden to the observed delay in disease progression for the group of animals in the vaccine study.

Anamnestic responses to virus challenge in immunized ponies

Anamnestic cellular and humoral immune responses after virus challenge were measured in animals receiv-

TABLE 1

Kinetics of Virus Replication in Vaccinated and Control Ponies Challenged with $EIAV_{PV}$

Pony no.	Plasma viremia level on day of challenge	Viral RNA levels (molecules/ml)[a]				Day postchallenge of initial viremia[c]	Day postchallenge of initial EIA fever[d]
		Day of challenge	7 days p.i.[b]	14 days p.i.	21 days p.i.		
579	Neg.[e]	$<10^{2}$[f]	3×10^{2}	8×10^{2}	5×10^{3}	16	N/A[g]
621	Neg.	$<10^{2}$	$<10^{2}$	8×10^{2}	1×10^{3}	>22[h]	44
645	Neg.	$<10^{2}$	6×10^{2}	1×10^{4}	1×10^{5}	14	18
649	Neg.	$<10^{2}$	1×10^{2}	5×10^{2}	1×10^{3}	15	58
Control ponies[i]							
6	N/A[j]	N/A	$<10^{2}$	2×10^{3}	2×10^{7}	13	19
49	N/A	N/A	$<10^{2}$	4×10^{3}	5×10^{5}	11	18
98	N/A	N/A	2×10^{5}	1×10^{8}	1×10^{7}	8	21
561	N/A	N/A	$<10^{2}$	2×10^{6}	2×10^{6}	11	16
562	N/A	N/A	$<10^{2}$	2×10^{6}	3×10^{5}	11	17
564	N/A	N/A	$<10^{2}$	8×10^{5}	2×10^{6}	12	18
567	N/A	N/A	$<10^{2}$	2×10^{6}	1×10^{6}	11	18

[a] Viral RNA molecules per milliliter were calculated by semiquantitative RT-PCR.
[b] Days postinoculation.
[c] Plasma collected this day post virus inoculation resulted in a positive RT measurement when incubated undiluted on fetal equine kidney cell cultures.
[d] The first day post virus challenge during which a rectal temperature above 39°C was measured.
[e] Plasma collected on the day of challenge resulted in no measureable RT activity when incubated undiluted on fetal equine kidney cell cultures.
[f] The lower limit of the assay was determined to be 10^{2} molecules per ml.
[g] This pony did not have a temperature above 39°C during the study.
[h] No samples were tested after 22 days post virus inoculation.
[i] Ponies listed were nonvaccinated and challenged with $EIAV_{PV}$ (Hammond *et al.,* 1997; Raabe *et al.,* 1998).
[j] Does not apply (N/A).

ing the particulate vaccine compared with nonvaccinated animals. EIAV-specific CTL were not readily observed on the day of virus challenge in three of four animals immunized with the particulate vaccine regimen (Fig. 5B). In contrast, *env*- and/or *gag*-specific CTL was readily detected in two of four vaccinated animals (621 and 649) 9 weeks after virus challenge (Fig. 5C). Moreover, statistically significant but very low levels of CTL were observed in the other two vaccinated ponies in the study (Fig. 5C). The levels of CTL activity observed in the vaccinates were consistent with the transient levels of EIAV-specific CTL activity (0–40% specific lysis) measured within the first 9 weeks after virus challenge in nonvaccinated ponies receiving the same amount of infectious virus (Hammond *et al.,* 1997).

Interestingly, the anamnestic humoral immune responses differed markedly from the cell-mediated immune responses in the vaccinates in that EIAV-specific antibody both quantitatively and qualitatively equaled or surpassed the measured levels in nonvaccinated virus infected ponies. The *env*-specific antibody levels in the vaccinates increased rapidly in the 3 weeks after introduction of infectious virus (Fig. 6A). By 6 weeks after virus challenge, quantitative levels of *env*-specific antibody reached a maximum in a similar manner as nonvaccinated animals. Although the vaccinated ponies were primed with antigen before virus challenge, the priming with EIAV-beads did not induce quantitatively a

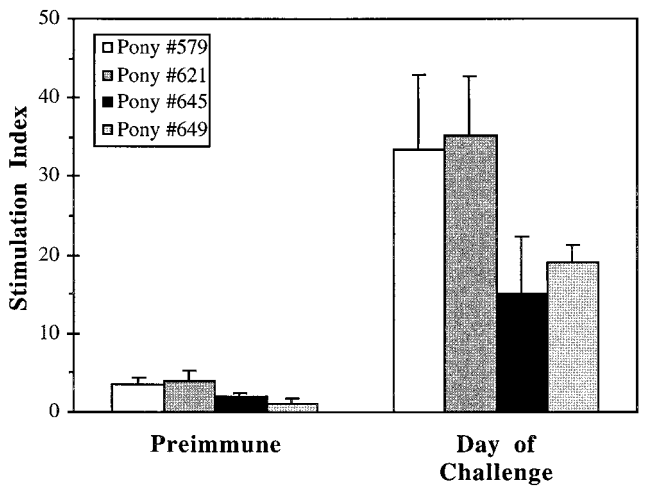

FIG. 4. *In vitro* lymphoproliferative responses of PBLs isolated from EIAV-bead vaccinated ponies to EIAV-infected autologous macrophages. PBMC samples collected longitudinally from the four vaccinated outbred ponies (579, 621, 645, and 649) were analyzed for lymphoproliferative responses to autologous macrophages infected with $EIAV_{PV}$, as described under Materials and Methods. The lymphoproliferative reactivities were presented in the form of an SI representing [^{3}H]thymidine incorporation of antigen-stimulated cultures divided by [^{3}H]thymidine incorporation of unstimulated cultures. Mean incorporation of [^{3}H]thymidine of replicate unstimulated lymphocyte cultures were always <400 cpm. All experiments were conducted in quadruplicate. The *y*-axis error bars represent the SI S.E.M. as calculated under Materials and Methods.

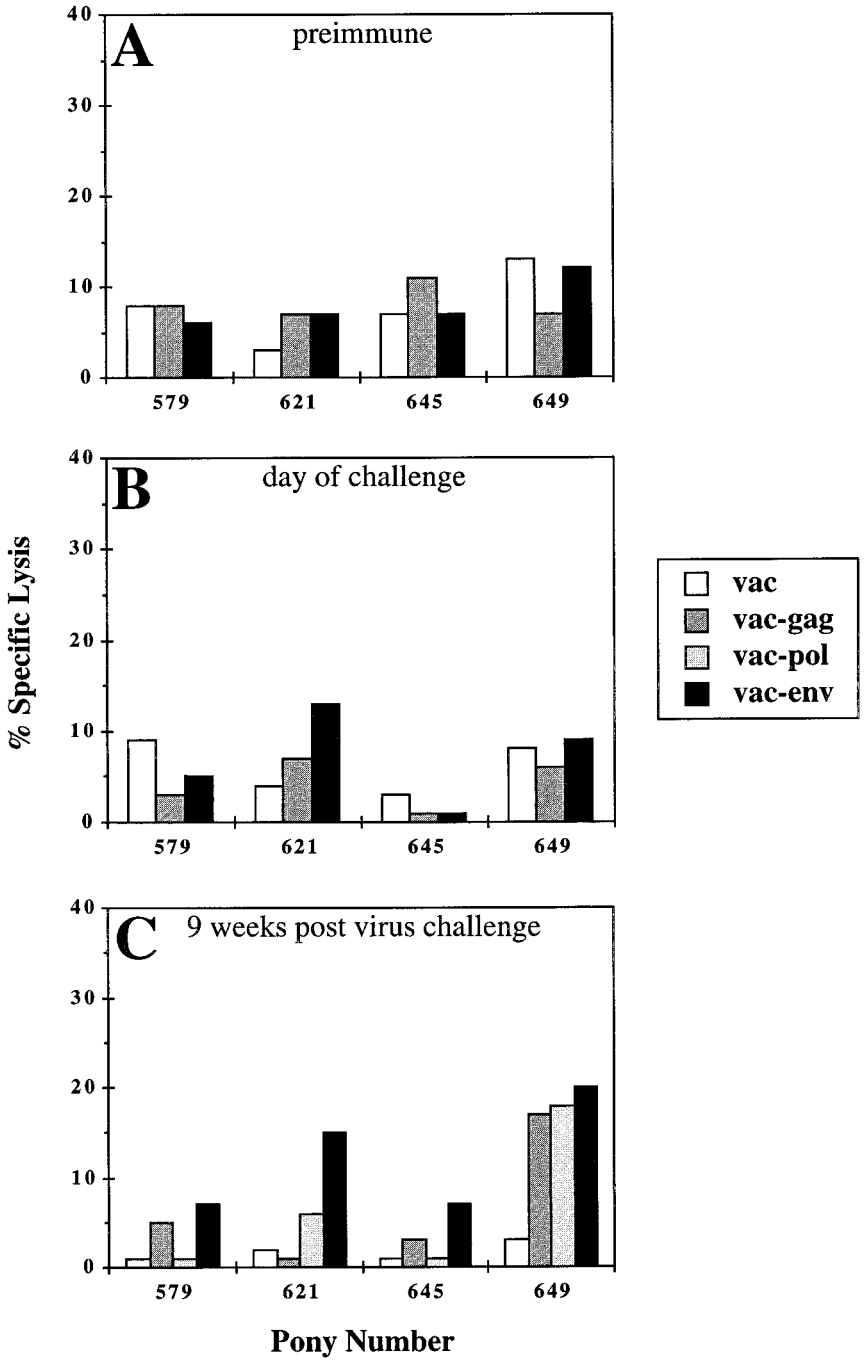

FIG. 5. CTL activity present in ponies before vaccination, at the day of virus challenge, and at 9 weeks postchallenge. PBLs isolated from long-term infected ponies before administration of the vaccine (A), on the day of virus challenge (B), or 9 weeks after virus challenge (C) were activated and expanded *in vitro* in parallel with one of two methods: autologous macrophages infected with EIAV or autologous PWM-stimulated lymphoblasts infected with three separate recombinant vaccinia virus vectors carrying sequences encoding EIAV *gag*, *pol*, and *env*. The responding cultures were used at an effector-to-target ratio of 30:1 in a standard chromium release assay after depletion of $CD4^+$ cells to enrich for $CD8^+$ T cells. Autologous target cells were generated by infection with recombinant vaccinia virus vectors carrying sequences encoding β-galactasidase (left bars), EIAV *gag* (second from the left), *env* (third from the left), or *pol* (right bars). NK cell lytic activity was not observed on using nonautologous target cells in the chromium release assays (data not shown). Data presented are representative of three separate experiments. The S.E.M. for all data points was always $<3\%$.

more rapid *env*-specific humoral immune response to infectious EIAV than nonvaccinates. Similar temporal anamnestic responses in vaccinates and nonvaccinates were observed when measuring the stability of the antigen–antibody complexes in an avidity ELISA (Fig. 6C). In contrast to the lack of differences between the vaccinates and nonvaccinates in the quantitative amounts of *env*-specific antibody and avidity measurements, striking

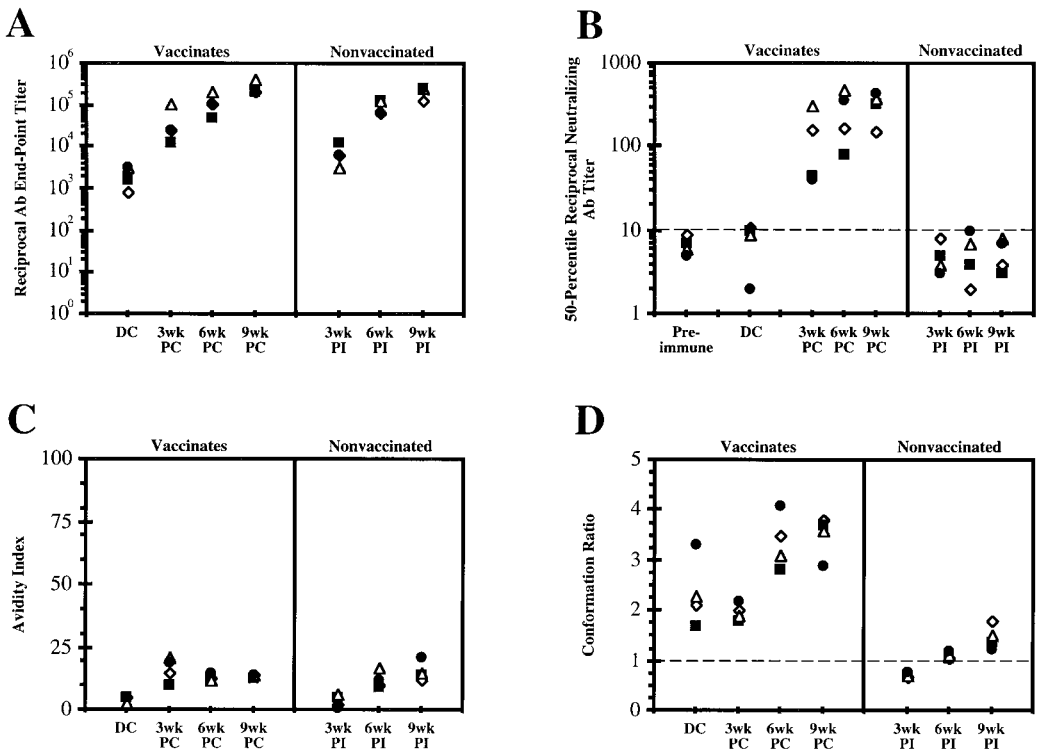

FIG. 6. Quantitative and qualitative measurements of serum antibodies from vaccinated ponies specific for the EIAV envelope glycoprotein. Serum IgG reactivity to EIAV envelope glycoproteins was measured in a concanavalin A ELISA as described under Materials and Methods. Each panel was separated into two parts for rapid visual comparison. Sera were collected at the day of challenge (DC), 3 weeks after virus challenge (3wk PC), 6 weeks after virus challenge (6wk PC), and 9 weeks after virus challenge (9wk PC) from outbred ponies vaccinated with the EIAV-beads vaccine (left) and collected at 3 weeks postinfection (3wk PI), 6 weeks postinfection (6wk PI), and 9 weeks postinfection (9wk PI) from nonvaccinated control animals infected with EIAV (right). Results are the averages of at least two independent assays. Data from ponies 579 (◇), 621 (■), 645 (△), and 649 (●) were presented on the vaccinated half of each panel. Data from ponies 561(◇), 562 (■), 564 (△), and 567 (●), which had been experimentally infected with $EIAV_{PV}$, were presented on the nonvaccinated half of each panel. (A) Reciprocal end point titer quantification of EIAV envelope-specific IgG present in serum collected at various time points from vaccinated and nonvaccinated ponies that had been challenged with infectious EIAV. (B) The 50% reciprocal neutralizing antibody titer measured in serum collected at various time points from vaccinated and nonvaccinated ponies challenged with infectious EIAV. The S.D. was always <6%. (C) The avidity index of EIAV envelope-specific IgG present in serum collected at various time points from vaccinated and nonvaccinated ponies challenged with infectious EIAV. (D) The conformation dependence of EIAV envelope-specific IgG present in serum collected at various time points from vaccinated and nonvaccinated ponies challenged with infectious EIAV. A conformation ratio value of >1.0 (dotted line) signifies a predominance of the antibody population specific for conformation dependent epitopes, whereas a conformation ratio of <1 signifies a predominance of the antibody population specific for linear epitopes.

differences were observed qualitatively (neutralization activity and conformational epitope dependence), with the *env*-specific antibody in the ponies receiving the EIAV-beads vaccine compared with nonvaccinates. Neutralizing antibody activity present in the serum of vaccinates rapidly increased to high levels of >1:300 dilution for a 50% neutralizing antibody titer just 3 weeks after virus challenge (Fig. 6B). This observation is in sharp contrast to the lack of neutralizing antibody (<1:10) even at 9 weeks postinfection in nonvaccinated animals (Fig. 6B). Similarly, the predominance of conformational epitopes of the *env*-specific antibody populations in vaccinated animals present at the day of challenge increased markedly from an average of 2.3 to 3.5 during the time of observation (Fig. 6D). In contrast, *env*-specific antibody responses in naive ponies were initially directed to linear determinants (at 3 weeks postinfection, conformation ratio was 0.7) and became predominantly conformational (ratio >1) after 6 weeks postinfection (Fig. 6D). Thus the EIAV-beads preparation appeared to induce an *env*-specific antibody population adept at recognizing and responding more rapidly to conformationally dependent and neutralizing epitopes present on virions. Taken together, this vaccine regimen, although not eliciting a detectable virus-specific CTL response, induced positive immunological responses that delayed disease progression, reduced virus burden, and provided a rapid anamnestic humoral response highlighted by considerable amounts of virus neutralizing activity.

DISCUSSION

We present here the first study using a particulate immunization strategy in a large animal model. Our results demonstrate that a virus particulate vaccine, while not preventing infection, reduced the level of virus repli-

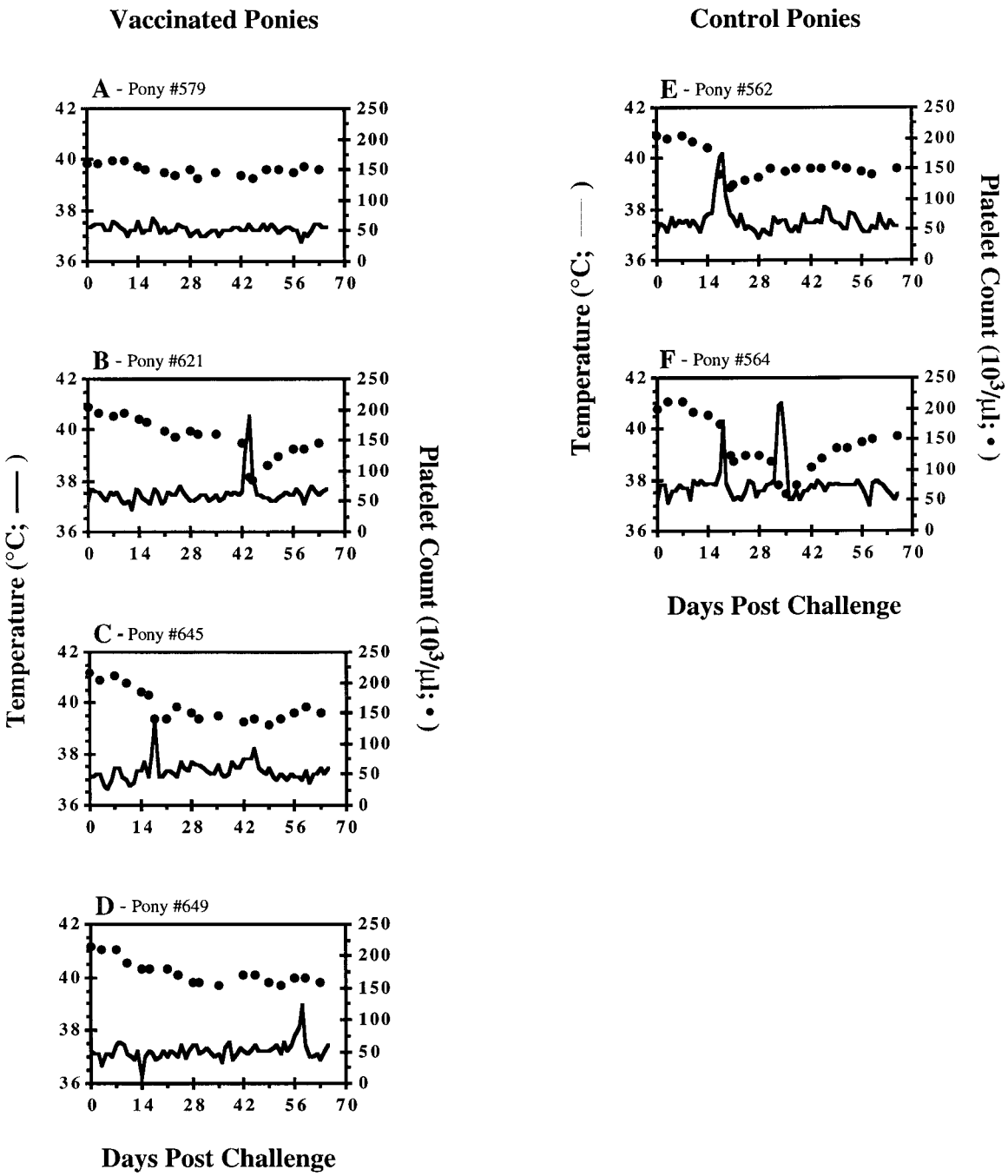

FIG. 7. Clinical profiles of vaccinated ponies after challenge with homologous infectious EIAV. Rectal temperatures (solid line, primary *y*-axis) and platelet counts per microliter of whole blood (●, secondary *y*-axis) of four outbred ponies (A–D; ponies 579, 621, 645, and 649) that had been inoculated intradermally with a total of 600 μg of EIAV attached to iron oxide beads before challenge on day 0 with infectious $EIAV_{PV}$ and two representative control ponies (E and F; ponies 562 and 564) that had not been immunized but were experimentally infected with $EIAV_{PV}$. Rectal temperatures in excess of 39°C were considered specific EIA-related episodes.

cation and delayed the development of disease in three of four immunized ponies challenged with homologous virus. Indeed, we established a statistically significant correlation between levels of virus replication and delay of disease progression in the vaccinated ponies compared with nonvaccinated ponies. In general, the vaccinated ponies with a delay in disease progression had on average up to 1000-fold lower viral RNA levels in the plasma at days 14 and 21 after virus challenge than the control ponies. Taken together, our findings indicate that the particulate vaccine regimen elicited immune responses providing partial protection for ponies on challenge with infectious virus. Although we demonstrated a level of protection induced by the vaccine regimen, these

studies do not identify any obvious immune correlates of protection at the day of challenge. However, within 3 weeks after virus challenge, anamnestic humoral responses characterized by a rapid emergence of neutralizing activity in the serum and a predominance of antibodies to conformationally dependent epitopes were observed in the vaccinates, indicating a beneficial priming effect elicited by the particulate vaccine that could be measured *in vitro*.

Previous evaluations of candidate EIAV vaccines to elicit protective immune responses against challenge with pony virulent virus reveal a broad spectrum of vaccine efficacy in preventing EIAV infection and the development of disease (Montelaro *et al.,* 1996). Ponies immunized with a baculovirus-expressed recombinant envelope glycoprotein subunit (rgp90) vaccine and subsequently challenged with pony virulent virus produced a range of vaccine efficacies ranging from lack of clinical disease to severe enhancement (Raabe *et al.,* 1998). The rgp90 trial was similar to this study because all ponies in both trials had measurable viral RNA levels in their plasma. However, the rgp90 immunogen elicited severe enhancement of infection in some vaccinated ponies that was not observed in our particulate vaccine study. Ponies vaccinated with an inactivated whole virus or a lectin affinity-purified viral envelope glycoprotein were sterilely protected against homologous avirulent challenge (Issel *et al.,* 1992). However, only the whole virus vaccine, and not the glycoprotein vaccine, was able to protect ponies against infection on challenge with heterologous pony virulent virus. At the day of challenge, both the whole virus and glycoprotein vaccinates, as well as the particulate vaccinates in this study, had no detectable neutralizing antibody. Interestingly, anamnestic neutralizing antibody responses to the challenge virus strain were observed in animals that received the particulate vaccine but not the inactivated whole virus vaccine. Therefore, detectable anamnestic virus neutralizing antibody may not be a necessary component of a protective immune response to prevent establishment of infection on challenge by exogenous virus.

Particulate vaccines are noted for their ability to prime a vigorous antigen-specific CTL response *in vivo* (Falo *et al.,* 1995). Nevertheless, by day of challenge our particulate vaccine induced a vigorous virus-specific lymphoproliferative response with little measurable CTL activity. Our initial *in vitro* studies using EIAV covalently associated with iron oxide beads to prime antigen presenting cells demonstrated a potent virus-specific lymphoproliferative activity. Moreover, use of autologous macrophages primed with virus-coupled particulates to activate and expand virus-specific CTL *in vitro* led our group to believe that this priming mechanism would be very efficacious *in vivo*. We have considered several parameters that may have contributed to the absence of virus-specific CTL activity in animals receiving the EIAV-beads immunogen. First, this was the first study to analyze in a large animal model system the effectiveness of a particulate preparation to prime for viral immunity. Therefore it is conceivable that we did not immunize the animals with sufficient antigen preparation to effectively replicate the induction of immunity as in the murine model system. Second, the multicomponent nature of the immunogen could have diluted the levels of antigen below a threshold necessary to effectively prime antigen-specific CTL in sufficient quantities for measurement from peripheral blood mononuclear cells (PBMCs) in our *in vitro* assay. Third, our detection methods may not have been sensitive enough to accurately detect the virus-specific CTL induced by the particulate vaccine even though sufficient antigen was provided during the immunizations for an effective virus-specific CTL response to be generated systemically. Activating and expanding CTL in *in vitro* culture for 7 days is much less accurate in measuring antigen-specific CTL than direct measurements of cytokine production at the single cell level using flow cytometry or ELISPOT methods (Murali-Krishna *et al.,* 1998). Unfortunately, the reagents to directly measure interferon-γ or interleukin-4 production at the single cell level using cytokine-specific monoclonal antibodies in ELISPOT and/or flow cytometric analyses or MHC tetramer technology for direct quantification of antigen-specific T cells is not yet available in the equine system.

At the day of challenge, the EIAV-beads regimen induced an "immature" humoral immune response of moderate titer, low avidity, and non-neutralizing activity, substantiating our previous studies suggesting that a mature immune response will be required for protective immunity (Cole *et al.,* 1997; Hammond *et al.,* 1997). Interestingly, anamnestic humoral immune responses after virus challenge were associated with an increase in antibody titer, the rapid emergence of strong neutralizing antibody activity, and the maintenance of the predominance of conformationally dependent *env*-specific epitopes. Strong anemnestic neutralizing antibody responses to SIV infection were similarly observed in macaques initially receiving cell-free replication-incompetent SIV intravenously or SIV in adjuvants intramuscularly and boosted subcutaneously with particulate SIV (Kraiselburd *et al.,* 1997). These observations suggest the potential effectiveness of a particulate vaccination regimen to induce antibody responses specific for proteins in a more native form, whereas adjuvanted antigens may present epitopes in a more denatured or masked format. Our observations indicate that the effective production of neutralizing antibodies to lentiviral envelope glycoproteins may depend on the qualitative means by which the antigen is presented to the humoral arm of the immune system.

In this initial study, EIAV-beads vaccine regimen did not induce protective or sterile immunity to virus exposure. Several parameters could be evaluated more thor-

oughly to provide a more effective vaccine regimen in this large animal model. These parameters include the amount of viral protein at each inoculation, the number of immunizations, and the period of immunization before virus challenge. Moreover, further studies using selected EIAV subunit antigens coupled to particulate beads instead of a multicomponent virus particle may prove to be a more effective approach. Clearly, more studies using particulate antigens should be conducted in various large animal model systems to properly develop and use this vaccination strategy as a means to induce protective immunity.

MATERIALS AND METHODS

EIAV strains

Three reference strains of EIAV were used in this study. $EIAV_{Pr}$ is the prototype, nonpathogenic, cell culture-adapted strain of EIAV initially derived by cell adaptation of the Wyoming strain of EIAV (Malmquist *et al.,* 1973). $EIAV_{PV}$ is a pathogenic and antigenic variant derived from $EIAV_{Pr}$ (Rwambo *et al.,* 1990). $EIAV_{WSU5}$ is a virulent strain of EIAV generated using procedures described to produce $EIAV_{PV}$ (McGuire *et al.,* 1994). These three strains of EIAV are very closely related, having $<1\%$ divergence at the amino acid level.

Experimental subjects, clinical evaluation, and sample collection

Four outbred ponies were selected for this study. All ponies were clinically monitored daily and maintained as described previously (Issel *et al.,* 1992). Rectal temperatures and clinical status were recorded daily. Samples of serum, plasma, and whole blood were collected from each pony at predetermined intervals. Plasma samples were collected at predetermined specified intervals and during each febrile episode ($>39°C$). Plasma samples were stored at $-80°C$ until used to determine the quantity of circulating infectious EIAV and of plasma RNA levels. Serum samples were stored at $-20°C$ until tested for antibody reactivity in an ELISA, Western blot analysis, or a neutralization test. Whole blood samples were appropriately fractionated for enumeration of platelets (Unopette microcollection system; Becton-Dickinson, Rutherford, NJ) or experimentation with PBMCs. PBMCs were either used the same day for the evaluation of EIAV-specific proliferative responses and EIAV-specific cytolytic activity or stored in liquid nitrogen for reevaluation.

Antigen preparation and vaccination procedure

Gradient-purified $EIAV_{PV}$ was produced as described previously (Montelaro *et al.,* 1982). A particulate form of gradient-purified $EIAV_{PV}$ was constructed by covalently coupling virus to glutaraldehyde-activated 0.5- to 1.5-μm iron oxide beads (Advanced Magnetics, Cambridge, MA) as described previously (Falo *et al.,* 1995). The quantity of virus bound to the particulates was indirectly inferred by a subtractive measurement of the amount of virus originally in the preparation from the amount of virus remaining after covalent conjugation to the beads as determined with a Lowry-based assay (BioRad). Where indicated, gradient-purified EIAV was formaldehyde inactivated as described previously (Issel *et al.,* 1992), and gradient-purified EIAV bound to iron oxide particulates was subjected to 0.8% formaldehyde in $1\times$ PBS for 18 h at 4°C, neutralized with 2% sodium bisulfite in $1\times$ PBS, and extensively washed with 1X PBS before use in *in vitro* or *in vivo* experiments. Animals were inoculated on each side of the neck intradermally with a total of 200 μg of $EIAV_{PV}$ covalently coupled to iron oxide beads in saline. Each pony received a total of three inoculations at intervals of 3 weeks. All animals were challenged with 300 equid infectious doses of $EIAV_{PV}$ 9 weeks after receiving the initial injection of vaccine.

Micro-reverse transcriptase assay

The measurement of reverse transcriptase activity in the cell supernatants was conducted using a micro-reverse transcriptase assay for EIAV developed in this laboratory (Lichtenstein *et al.,* 1995) and described elsewhere (Raabe *et al.,* 1998). Macrophage and equine dermal cells used to culture virus were isolated and maintained as described previously (Raabe *et al.,* 1998).

Semiquantitative RT-PCR measurement of plasma virus levels

Plasma samples from all ponies were analyzed for the presence of viral RNA using a semiquantitative RT-PCR assay that used *gag*-specific amplification primers (Lichtenstein *et al.,* 1995). All samples were conducted in a minimum of two independent experiments.

Quantitative and qualitative serological assays

Serum IgG antibody reactivities to EIAV envelope glycoprotein were quantitatively (end point titer) and qualitatively (avidity index, conformation ratio) measured using a concanavalin A-based ELISA method as described (Hammond *et al.,* 1997). Virus neutralizing activity mediated by immune sera was assessed in an indirect cell ELISA-based, infectious center assay using a constant amount of infectious $EIAV_{PV}$ and sequential 2-fold dilutions of serum (Hammond *et al.,* 1997).

Lymphocyte proliferation assays

EIAV-specific proliferative responses were measured by using autologous $EIAV_{PV}$-infected monocytes as antigen presenting cells (Hammond *et al.,* 1997). EIAV-specific lymphocyte proliferative responses were reported in

the form of a stimulation index (SI) representing the average [^{3}H]thymidine incorporation of replicate EIAV-infected monocyte stimulated lymphocyte cultures (E) divided by the average [^{3}H]thymidine incorporation of replicate unstimulated lymphocyte cultures (M), or SI = E/M. Median incorporation of [^{3}H]thymidine of replicate unstimulated lymphocyte cultures were always <400 cpm. The SI ratio S.E.M. was calculated as defined previously (Hammond *et al.,* 1997).

Recombinant vaccinia virus vectors

The control recombinant vaccinia virus vector (vac) has been described in detail (vSC8; Chakrabarti *et al.,* 1985). The recombinant vaccinia virus vector carrying the full-length $EIAV_{WSU5}$ *env* gene (vac-*env*) has been characterized previously (vENV2; McGuire *et al.,* 1994). The recombinant vaccinia virus vectors carrying the full-length $EIAV_{Pr}$ *gag* (vac-*gag*) and *pol* (vac-*pol*) were derived from the $EIAV_{Pr}$ proviral clone (accession no. M16575). Target cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 10 for 12–18 h at 37°C. More than 99% of the equine target cells infected with recombinant vaccinia viruses expressed antigen as determined by indirect immunofluorescence (data not shown).

CTL assay

Cytolytic activity was measured in a standard ^{51}Cr-release assay developed for the equine model system (Hammond *et al.,* 1997, 1998). PBMCs were isolated from ponies at designated intervals postvaccination or after virus challenge by discontinuous density gradient centrifugation. PBMCs were activated and expanded *in vitro* in parallel with one of two types of stimulator cells: autologous macrophages infected with EIAV (Hammond *et al.,* 1997)or autologous PWM-stimulated lymphoblasts infected with three individual recombinant vaccinia virus vectors carrying sequences encoding EIAV *gag*, *pol*, and *env* (Hammond *et al.,* 1998). *In vitro* depletion of $CD4^+$ T cells was conducted using the method of indirect panning (McGuire *et al.,* 1994). Generally, >95% of the $CD4^+$ or $CD8^+$ T cells were depleted as determined by flow cytometry analysis (data not shown). All experiments were performed in quadruplicate. The percent specific lysis was calculated as follows:

$$\%\ \text{Specific lysis} = \frac{(E - C)}{(N - C)} \times 100$$

where E is the average cpm from experimental wells containing T cells and target cells, C is the average cpm from control wells containing target cells only (spontaneous lysis), and N is the average cpm from control wells containing target cells in 0.5% Nonidet P-40 (maximal lysis). Spontaneous lysis of the target cells was always below 30% of maximal lysis. The percent specific lysis S.E.M., determined as described previously (Siliciano *et al.,* 1985), was always <3%.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grant 5RO1-AI25850 from the National Institutes of Allergy and Infectious Disease, by funds from the Lucille Markey Charitable Trust, and by the Kentucky Agricultural station. S.A.H. was supported by National Institutes of Health AIDS Training Grant 5T32-AI07487. We thank Ms. Kathleen Thompson (Department of Dermatology, University of Pittsburgh School of Medicine) for technical assistance in the preparation of the particulate antigens, Dr. Walter Storkus (Department of Medicine, University of Pittsburgh School of Medicine) for providing the recombinant human interleukin-2, Dr. Travis McGuire (Department of Veterinary Microbiology and Pathology, Washington State University) for generously providing the vENV2 construct, Dr. Joseph Ahearn (Department of Rheumatology, School of Medicine, University of Pittsburgh) for use of the Beckton Dickenson FACSCalibur flow cytometer, Drs. Sekhar Chakrabarti and Bernard Moss (NIAID and the NIH AIDS Research and Reference Reagent Program) for the recombinant vaccinia virus vector vSC8, and Mr. Gary Thomas and Mr. Brian Meade for their maintenance of the animal facility and care of the animals.

REFERENCES

Chakrabarti, S., Brechling, K. B., and Moss, B. (1985). Vaccinia virus expression vector: Coexpression of beta-galactosidase provides visual screening of recombinant virus plaques. *Mol. Cell. Biol.* **5,** 3403–3409.

Cole, K. S., Rowles, J. L., Murphey-Corb, M., Clements, J. E., Unangst, T., Robinson, J., Desrosiers, R. C., Wyand, M. S., and Montelaro, R. C. (1997). Evolution of envelope-specific antibody responses in monkeys experimentally infected or immunized with SIV and its association with the development of protective immunity. *J. Virol.* **71,** 5069–5079.

Falo, L. D., Jr., Kovacsovics-Bankowski, M., Thompson, K., and Rock, K. L. (1995). Targeting antigen into the phagocytic pathway *in vivo* induces protective tumour immunity. *Nat. Med.* **1,** 649–653.

Hammond, S. A., Cook, S. J., Lichtenstein, D. L., Issel, C. J., and Montelaro, R. C. (1997). Maturation of the cellular and humoral immune responses to persistent infection in horses by equine infectious anemia virus is a complex and lengthy process. *J. Virol.* **71,** 3840–3852.

Hammond, S. A., Issel, C. J., and Montelaro, R. C. (1998). General method for the detection and in vitro expansion of equine cytolytic T lymphocytes. *J. Immunol. Methods* **213,** 73–85.

Issel, C. J., and Foil, L. D. (1984). Studies on equine infectious anemia virus transmission by insects. *J. Am. Vet. Med. Assoc.* **184,** 293–297.

Issel, C. J., Horohov, D. W., Lea, D. F., Adams, W. V., Jr., Hagius, S. D., McManus, J. M., Allison, A. C., and Montelaro, R. C. (1992). Efficacy of inactivated whole-virus and subunit vaccines in preventing infection and disease caused by equine infectious anemia virus. *J. Virol.* **66,** 3398–3408.

Kovacsovics-Bankowski, M., and Rock, K. L. (1994). Presentation of exogenous antigens by macrophages: analysis of major histocompatibility complex class I and class II presentation and regulation by cytokines. *Eur. J. Immunol.* **24,** 2421–2428.

Kraiselburd, E., Salaman, A., Beltrán, M., Rivera, M., Oliver, J., Kessler, M., Knezevich, M., Rodriguez, A., Bilska, M., Montefiori, D., Torres-Bauza, L., and Martinez, I. (1997). Vaccine evaluation studies of replication-defective SIVsmB7. *Cell. Mol. Biol.* **43,** 915–924.

Lichtenstein, D. L., Rushlow, K. E., Cook, R. F., Raabe, M. L., Swardson, C. J., Kociba, G. J., Issel, C. J., and Montelaro, R. C. (1995). Replication in vitro and in vivo of an equine infectious anemia virus mutant deficient in dUTPase activity. *J. Virol.* **69,** 2881–2888.

Malmquist, W. A., Barnett, D., and Becvar, C. S. (1973). Production of equine infectious anemia antigen in a persistently infected cell line. *Arch. Virol.* **42,** 361–370.

McGuire, T. C., Tumas, D. B., Byrne, K. M., Hines, M. T., Leib, S. R., Brassfield, A. L., O'Rourke, K. I., and Perryman, L. E. (1994). Major histocompatibility complex-restricted $CD8^+$ cytotoxic T lymphocytes from horses with equine infectious anemia virus recognize *env* and *gag*/pr proteins. *J. Virol.* **68,** 1459–1467.

Montelaro, R. C., Ball, J. M., and Rushlow, K. E. (1993). Equine retroviruses. *In* "The Retroviridae" (J. Levy, Ed.), Vol. 2**,** pp. 257–360. Plenum Press, New York.

Montelaro, R. C., Grund, C., Raabe, M., Woodson, B., Cook, R. F., Cook, S., and Issel, C. J. (1996). Characterization of protective and enhancing immune responses to equine infectious anemia virus resulting from experimental vaccines. *AIDS Res. Human Retroviruses* **12,** 413–415.

Montelaro, R. C., Lohrey, N., Parekh, B., Blakeney, E. W., and Issel, C. J. (1982). Isolation and comparative biochemical properties of the major internal polypeptides of equine infectious anemia virus. *J. Virol.* **42,** 1029–1038.

Murali-Krishna, K., Altman, J. D., Suresh, M., Sourdive, D. J. D., Zajac, A. J., Miller, J. D., Slansky, J., and Ahmed, R. (1998). Counting antigen-specific CD8 T cells: A reevaluation of bystander activation during viral infection. *Immunity* **8,** 177–187.

Raabe, M., Issel, C., Cook, S., Cook, R., Woodson, B., and Montelaro, R. (1998). Immunization with a recombinant envelope protein (rgp90) of EIAV produces a spectrum of vaccine efficacy ranging from lack of clinical disease to severe enhancement. *Virology* **245,** 151–162.

Raabe, M. R., Issel, C. J., and Montelaro, R. C. (1998). Equine monocyte-derived macrophage cultures and their applications for infectivity and neutralization studies of equine infectious anemia virus. *J. Virol. Methods* **71,** 87–104.

Rock, K. L. (1996). A new foreign policy: MHC class I molecules monitor the outside world. *Immunol. Today* **17,** 131–137.

Rwambo, P. M., Issel, C. J., Hussain, K. A., and Montelaro, R. C. (1990). In vitro isolation of a neutralization escape mutant of equine infectious anemia virus (EIAV). *Arch. Virol.* **111,** 275–280.

Sellon, D. C., Fuller, F. J., and McGuire, T. C. (1994). The immunopathogenesis of equine infectious anemia virus. *Virus Res.* **32,** 111–138.

Siliciano, R. F., Keegan, A. D., Dintzis, R. Z., Dintzis, H. M., and Shin, H. S. (1985). The interaction of nominal antigen with T cell antigen receptors. I. Specific binding of multivalent nominal antigen to cytolytic T cell clones. *J. Immunol.* **135,** 906–914.

Stein, C. D., Lotze, J. C., and Mott, L. O. (1942). Transmission of equine infectious anemia by the stablefly, *Stomoxys calcitrans*, the horsefly, *Tabanus sulcifrons* (Macquart), and by injection of minute amounts of virus. *Am. J. Vet. Res.* **3,** 183–193.